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Effect of Genetically Modified Poplars on Soil Microbial Communities during the Phytoremediation of Waste Mine Tailings▽†

Moonsuk Hur,[1] Yongho Kim,[1] Hae-Ryong Song,[1] Jong Min Kim,[1] Young Im Choi,[2] and Hana Yi[3]*

Biosafety Research Team, Environmental Health Research Department, National Institute of Environmental Research, Incheon 404-708, Republic of Korea[1]; Biotechnology Division, Korea Forest Research Institute, Suwon, Kyunggi-do 441-350, Republic of Korea[2]; and Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Republic of Korea[3]

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The application of transgenic plants to clean up environmental pollution caused by the wastes of heavy metal mining is a promising method for removing metal pollutants from soils. However, the effect of using genetically modified organisms for phytoremediation is a poorly researched topic in terms of microbial community structures, despite the important role of microorganisms in the health of soil. In this study, a comparative analysis of the bacterial and archaeal communities found in the rhizosphere of genetically modified (GM) versus wild-type (WT) poplar was conducted on trees at different growth stages (i.e., the rhizospheres of 1.5-, 2.5-, and 3-year-old poplars) that were cultivated on contaminated soils together with nonplanted control soil. Based on the results of DNA pyrosequencing, poplar type and growth stages were associated with directional changes in the structure of the microbial community. The rate of change was faster in GM poplars than in WT poplars, but the microbial communities were identical in the 3-year-old poplars. This phenomenon may arise because of a higher rate and greater extent of metal accumulation in GM poplars than in naturally occurring plants, which resulted in greater changes in soil environments and hence the microbial habitat.

Heavy metal pollution threatening human health and the ecosystem is a growing concern worldwide (55). Excessive levels of heavy metals cause serious damage to living organisms that inhabit such environments (3). Furthermore, as a result of the bioaccumulation of heavy metals over time, organisms that do not inhabit contaminated areas may also be subject to higher exposure through the food chain (67). One of the main sources of heavy metal pollution in soil, water, and sediments is the metal purification procedure, which includes mining, smelting, and the tailings from these industries (55). In Korea, approximately 1,000 metal mines have been suspended or closed in the last 30 years, with metal mine tailings amounting to about 10 million tons (40a). The leftover mine tailings contain high concentrations of heavy metals and are a major source of environmental pollution; thus, a number of physicochemical methods have been developed to prevent pollution and/or restore the ecosystem of polluted sites (51).

Phytoremediation, which is the use of plants to clean up environmental pollution, has received much attention as a promising method for the removal of metal pollutants in soils (6, 66). Phytoremediation is a cost-effective and environmentally friendly approach compared to other environmentally invasive, expensive, and inefficient cleanup technologies (66). A number of plant species are capable of high-level organic compound degradation or heavy metal hyperaccumulation. Viable candidates for metal phytoremediation include the alpine pennycress *Thlaspi caerulescens*, the Indian mustard *Brassica juncea*, the sunflower *Helianthusannuus*, the yellow poplar *Liriodendrontulipifera*, and the shrub tobacco *Nicotianaglaucum* (6). However, slow rates of removal and incomplete metabolism have restricted the application of phytoremediation in the field (66). Thus, genetically engineered plants that exhibit enhanced performance with respect to the metabolism of toxic compounds have been developed by the overexpression and/or introduction of genes from other organisms (15, 21). In particular, transgenic poplars with enhanced uptake and metabolism of heavy metals have been developed (1, 5, 15, 58). In general, the genus *Populus* is an effective phytoremediator because of its rapid growth rates (3 to 5 m/year); moreover, it produces a large biomass within a short period of time of around 5 to 8 years (63, 71). For example, the transgenic poplar developed by the overexpression of the rabbit *P450 2E1* gene to create a hybrid poplar (*Populus tremula* × *P. alba*) was reported to show a 100-fold enhancement of phytoremediation capacity (15). In addition, the yellow poplar (*L. tulipifera*) expressing modified mercuric reductase (*merA*) showed elevated resistance to toxic levels of mercury (5, 58).

Engineered poplars have greatly increased the possibility of the practical application of phytoremediation. However, this technology is still in the developmental stage, with the field testing of transgenic plants for phytoremediation being very limited. The major obstacle is biosafety concern, because the unwanted effects of genetically modified organisms are still not fully understood. One of the most postulated potential risks of

* Corresponding author. Mailing address: Institute of Molecular Biology and Genetics, Seoul National University, 599 Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea. Phone: 82-2-876-8153. Fax: 82-2-874-8153. E-mail: hanayi@gmail.com.

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genetically modified (GM) plants is alteration to the structure of indigenous microbial communities (68). Because soil microorganisms play significant roles in the global cycling of organic and inorganic matter, as well as the maintenance of soil structure (9), alteration in the diversity or activity of microbial communities may have adverse effects on soil ecology (39). Hence, the effect of GM plants on soil microbial communities remains highly controversial. Several studies have reported that microbial communities are clearly altered by engineered plants (2, 14, 28, 42, 44, 61, 62). In contrast, other studies have shown that the associated changes in microbial communities with engineered plants are statistically insignificant (16, 35, 40, 46) or very minor (12–14, 17, 27, 28, 38, 48, 59). Most of these studies have used non-sequencing based methods, such as community-level physiological profiles (CLPPs), fatty acid methyl ester (FAME), denaturing gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism analysis (T-RFLP). However, these fingerprinting methods are limited in their capacity to detect minor changes and the components of these changes. In addition, the number of clone sequences ($\leq$100 sequences per sample) surveyed in a few studies (40, 42, 44) is insufficient to determine overall community profiles. Thus, a systematic investigation using next-generation sequencing (NGS) technology is necessary for the precise assessment of microbial community dynamics associated with GM plants.

Plant-microbe interactions in the rhizosphere affect the efficiency of metal phytoremediation (6). This is because microorganisms enhance the mobility and availability of metals to the plant by the release of chelating agents, acidification, phosphate solubilization, and redox changes (60). Moreover, many anaerobic microorganisms may independently remove a number of metals from the environment by reducing them to a lower redox state (43, 47). Thus, the microbial community structure associated with metal phytoremediation is of great interest, with DGGE and GeoChip analyses having been conducted for alpine pennycress, Scots pine, and poplar (18, 26, 56, 70). However, to date, no studies have used the NGS technique to survey the microbial community structure of highly metal-contaminated soil.

We sought here to evaluate the effect of genetically modified organisms (GMOs) on soil microbial communities during the phytoremediation of metal-contaminated soil. For this purpose, the rhizosphere soils of GM and wild-type (WT) poplars for a range of poplar growth stages (the rhizospheres of 1.5-, 2.5-, and 3-year-old poplars) were sampled together with nonplanted control soil and analyzed to determine the composition of the bacterial and archaeal communities by DNA pyrosequencing. The results improve our understanding of the effect of genetically modified poplars on the environment.

MATERIALS AND METHODS

Site description and soil samples. The study site was the trial station of the Korea Forest Research Institute, Korea Forest Service, where the phytoremediation of heavy metals was investigated using GM poplars of a nonflowering mutant hybrid (*P. alba* × *P. tremular* var. *glandulosa*). The trial station is located at Socheon-myeon, Bonghwa-gun, Gyeongsangbuk-do Province, Korea (129°3′17.343″E, 36°51′23.535″N, 620 m above sea level). Until about 10 years ago, this station was filled with zinc mine tailings, the top layer of which was covered with noncontaminated soil. The area of the trial station is 10,000 m^2 (1 ha), and the area of planted WT and GM poplars together covered 1,000 m^2 (0.1 ha).

In early July 2010, soil samples were taken from five sampling points within each of eight experimental sites (40 sampling locations in total). The first and second control sites, namely, CI and CII, were two independent sites of metal-contaminated soils without poplars. The area of each CI and CII was 100 m^2. Within each control site, the five sampling points were positioned 5 m apart from one another. The third, fourth, and fifth sites, namely, WT1.5, WT2.5, and WT3, comprised rhizosphere soils of WT poplars aged 1.5, 2.5, and 3 years, respectively. The remaining three sites, namely, GM1.5, GM2.5, and GM3, comprised rhizosphere soils of GM poplars aged 1.5, 2.5, and 3 years, respectively. The area of each experimental site was 3.6 m^2 and 5 m apart from each other. Within each experimental site, five replicate samples were taken from five different trees 0.6 m apart from each other. The soil within 30 cm from a main root was regarded as the rhizosphere, because rootlets measured in the present study extend up to 30 cm from the main root. The lengths of the main roots of 1.5-, 2.5-, and 3-year-old poplars were about 50 cm, 100 cm, and >100 cm, respectively. At each sampling point, 500 g of soil was collected from both the upper (2 to 15 cm) and lower (16 to 30 cm) layers. The soil was then sieved to select particles of < 2 mm in size, and individual soil samples were prepared by mixing the upper and lower soil layers of each sampling point. The bacterial community was analyzed using 40 individual samples belonging to all eight soil sites (CI, CII, WT1.5, WT2.5, WT3, GM1.5, GM2.5, and GM3), and the archaeal community was surveyed using seven composite samples representing seven of the sites (C, WT1.5, WT2.5, WT3, GM1.5, GM2.5, and GM3). The composite soil samples were prepared by mixing equal amounts of the five individual soil samples belonging to the corresponding soil site. The 40 individual soil samples and seven composite samples representing seven sites were kept on ice until sieving for subsequent analysis. The soil samples were stored at −80°C until DNA extraction.

Soil physicochemical analyses. For soil physicochemical analysis, the soils were air dried and further sieved to obtain <0.15-mm particles for metal analyses. A preliminary study showed that the study site was contaminated with high concentrations of As, Cd, Pb, and Zn, as well as low concentrations of Cr, Cu, Na, and Ni (data not shown). Thus, the total concentration of metals in the soil was determined for As, Cd, Pb, and Zn by using inductively coupled plasma atomic emission spectrometry (ICP-AES; Shimadzu) following the digestion with $HNO_3/HClO_4$ (4:1) (30). Soil pH, electrical conductivity (EC), total organic carbon content, and total nitrogen content were analyzed using standard methods (37, 52). Soil texture was analyzed by determining the percentage of sand, silt, and clay with a particle size analyzer (DE/QICPIC; Sympatec).

DNA extraction, PCR, and pyrosequencing. DNA was extracted from 1 g of soil sample with a commercial soil DNA isolation kit (MoBio). The extracted DNA was amplified using primers targeting the V1 to V3 regions of the prokaryotic 16S rRNA gene. The primers used for bacteria were V1-9F (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AC-GAGTTTGATCMTGGCTCAG-3′; underlining indicates the gene specific section) and V3-541R (5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-*X*-AC-WTTACCGCGGCTGCTGG-3′; the *X* barcode is uniquely designed for each subject, followed by a common linker [AC]). The primers used for archaeal organisms were AV1-21F (5′-CCTATCCCCTGTGTGCCTTGGCAGTC-TCAG-AG-TCCGGTTGATCCYGCCGG-3′) and AV3-519R (5′-CCATCTCATCCCTGCGTGTCTCCGAC-TCAG-*X*-GA-GGTDTTACCGCGGCKGCTG-3′). PCRs were carried out under the following conditions: initial denaturation at 94°C for 5 min, followed by 10 cycles of denaturation at 94°C for 30 s, annealing at 60°C to 55°C with a touchdown program for 45 s, and elongation at 72°C for 90 s. This was followed by an additional 20 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 45 s, and elongation at 72°C for 90 s. The amplified products were purified using resin columns (Qiagen), and 1 μg of PCR product for each subject was mixed and subjected to DNA pyrosequencing. The DNA sequencing was performed by Chunlab, Inc. (Seoul, Korea), with a Roche/454 GS FLX Titanium platform, according to the manufacturer's instructions.

Processing of sequencing data. The sequencing reads from the different samples were separated by unique barcodes. The sequences of the barcode, linker, and PCR primers were then removed from both sides of the original sequencing reads. The resultant sequences were subjected to a filtering process where only reads containing 0 to 1 ambiguous base calls (Ns) and 300 or more base pairs were selected for the final bioinformatics analyses. Nonspecific PCR amplicons that showed no match with the 16S rRNA gene database in a BLASTN search (expectation value of $>e^{-5}$) were also removed from the subsequent analyses. Chimeric sequences were detected by the analysis of differences in BLASTN-based sequence similarity patterns between the first half and second half of an object sequence. When the first and second halves were differentially identified

TABLE 1. Summary of sequencing data and diversity estimates[a]

Site	Bacteria (n = 5 for each site)				Archaea (n = 1 for each site)			
	Total reads	Subsampled reads	No. of species observed	Chao1 estimation	Total reads	Subsampled reads	No. of species observed	Chao1 estimation
Contaminated	7,229	1,300	221	354	11,611	1,800	24	30
WT1.5	4,064	1,127	218	350	8,526	1,800	27	31
WT2.5	5,264	1,300	421	836	12,242	1,800	30	50
WT3	3,864	1,300	675	1,575	11,942	1,800	32	64
GM1.5	5,514	1,300	403	741	1,845	1,800	29	29
GM2.5	6,164	1,300	547	1,188	8,848	1,800	28	33
GM3	6,183	1,300	641	1,431	9,205	1,800	29	32

[a] The values for bacteria are the arithmetic average for each site. The data for the archaea were obtained from seven mixed soil samples representing each site. "Total mixed read" values exclude chimeric and low-quality sequences (<300 bp and Ns ≥ 2).

at the bacterial order level, the sequence was regarded as a chimera and eliminated. For the taxonomic assignment of each pyrosequencing read, we used the EzTaxon-e database (http://www.eztaxon-e.org) (7), which contains 16S rRNA gene sequences of type strains that have valid published names and representative species-level phylotypes of either cultured or uncultured entries in the GenBank public database with complete hierarchical taxonomic classification from the phylum to the species level.

Statistical analyses. The diversity and species richness indices were calculated using the rRNA Database Project's pyrosequencing pipeline (http://pyro.cme.msu.edu/). The cutoff value for assigning a sequence to a species-level phylotype was ≥97% similarity. The cutoff value for the genus-level cluster was ≥94% similarity. In the event that the genus-level sequence cluster was not identified with a valid bacterial genus, the accession numbers of the GenBank sequence entry sharing the highest sequence similarity with the sequence cluster were used as the tentative generic name instead. Random subsampling was conducted to equalize data size, because the total number of reads that remained after preprocessing varied depending on samples. All of the statistical analyses were performed using this subset. The overall phylogenetic distance between each pair of communities was estimated using the Fast UniFrac web interface (29) and visualized using principal coordinate analysis (PCoA). The significance of differences among different sampling sites at the 0.05 significance level was detected with the Kruskal-Wallis rank sum test using R software. To detect the pairwise difference between each group, *post hoc* analysis was also performed with Tukey's HSD test (72). Tukey's HSD testing used the Familywise error rate to adjust the multiple testing problems. Canonical correspondence analysis (CCA) and one-way analysis of similarities (ANOSIM) were performed as implemented in PRIMER v6 (8).

RESULTS

Bacterial community composition. In the sample-size-normalized subsamples, the average number of bacterial species ranged from 218 to 675, depending on soil site (Table 1). The Chao1 estimator of species richness in the bacteria samples ranged from 350 to 1,575, indicating that ~2-fold more bacterial species were possibly present in the environment than were identified in the present study. Overall, the contaminated soils showed the lowest bacterial species richness, with higher microbial diversities being observed in the rhizospheres of older poplars. No bacterial species was characterized as being the exclusive (or dominant) taxon present in just the GM or WT site.

The overall composition of the bacterial DNA sequences amplified depended on the stage of poplar growth (Fig. 1a). Significant differences ($P < 0.001$) between soil sites were observed from ANOSIM, except for the two pairs comprising contaminated versus WT1.5, and WT3 versus GM3 (data not shown). The most striking feature was the abundance of sequences of the phyla AD3 (8.6%) and *Nitrospirae* (7.6%) in the contaminated soils, and their obvious decrement in poplar planted soil. The differential abundance of AD3 in contaminated soils compared to other soils was supported by a P value of 0.00004, but the significant difference of *Nitrospirae* was not observed ($P > 0.5$) because of the intersample variance. Together with AD3 and *Nitrospirae*, the sequences of species in the phyla *Acidobacteria*, *Firmicutes*, and *Cyanobacteria* decreased as the time period of poplar cultivation increased. In contrast, the proportion of *Bacteroidetes* and *Gemmatimonadetes* increased from 0.1% (contaminated soils) to 4% (older poplars). Together with *Bacteroidetes* and *Gemmatimonadetes*, the relative abundance of sequences assigned to the phyla *Proteobacteria*, *Actinobacteria*, and OP10 increased as the time period of poplar cultivation increased. Singularly, the phylum *Chloroflexi* predominated in the soils of 2.5-year-old WT poplars.

The representative bacterial genera that showed a tendency to change depending on the stage of poplar cultivation are summarized in Fig. SA1a in the supplemental material. The indicator genera of metal-contaminated soils were *Leptospirillum* ($P = 0.049$), *Acidithiobacillus* ($P = 0.086$), the cluster AD3 EU861914 (3.0E−05) and *Acidobacterium* ($P = 0.107$; the P value of *Acidobacterium* was calculated excluding WT1.5 sites). The relative sequence abundance of these 4 genera obviously decreased through the period of poplar phytoremediation. The clusters *Planctomyceteceae* DQ906072 ($P = 0.954$) and *Xanthomonadales* FJ228294 ($P = 0.004$) also showed a decreasing tendency as the age of the planted poplars increased. In contrast, no indicator genus was specified in the phyla *Bacteroidetes* and *Gemmatimonadetes*, where an apparent increasing tendency was observed at the phylum level. No genus belonging to these two phyla represented >1% of the total bacterial composition, even in the 3-year-old poplars. The increasing tendency of the phyla *Proteobacteria* and *Actinobacteria* was explained by an increase in the *Pseudolabrys*, *Arthrobacter*, and *Nocardioides* populations, in addition to the cluster *Rhizobiales* FM877535. The genera that were more noticeable in the middle stage of phytoremediation were the clusters *Ktedonobacteria* EU335272, *Ktedonobacterales* AM180160, *Thermosporotrichaceae* unclassified, *Methylophilales* AB452068, *Koribacter* EU335315, and *Acidobacteria* EF494368.

The UniFrac-based PCoA using 40 individual samples showed variation in the bacterial community associated with both poplar type (GM versus WT) and poplar age (Fig. 2).

Archaeal community composition. Archaeal diversity was as low as 24 to 32 species per soil site (Table 2). The overall

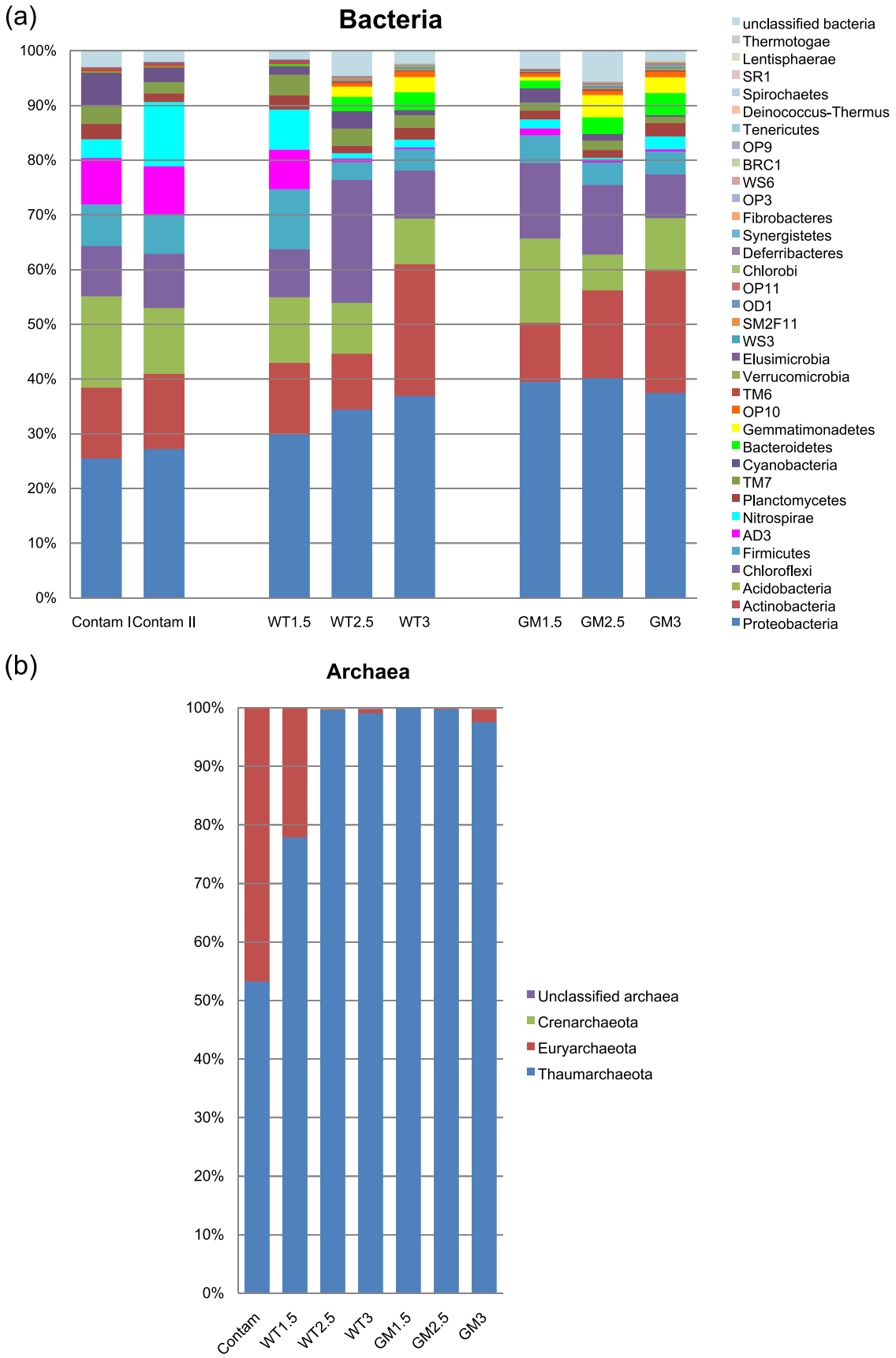

FIG. 1. Relative abundance of microbial phyla identified in contaminated, wild-type (WT), and genetically modified (GM) poplar soil sites. (a) Bacteria (sample, $n = 40$). Each column represents the average for five soil samples belonging to each site. (b) Archaea (sample, $n = 7$).

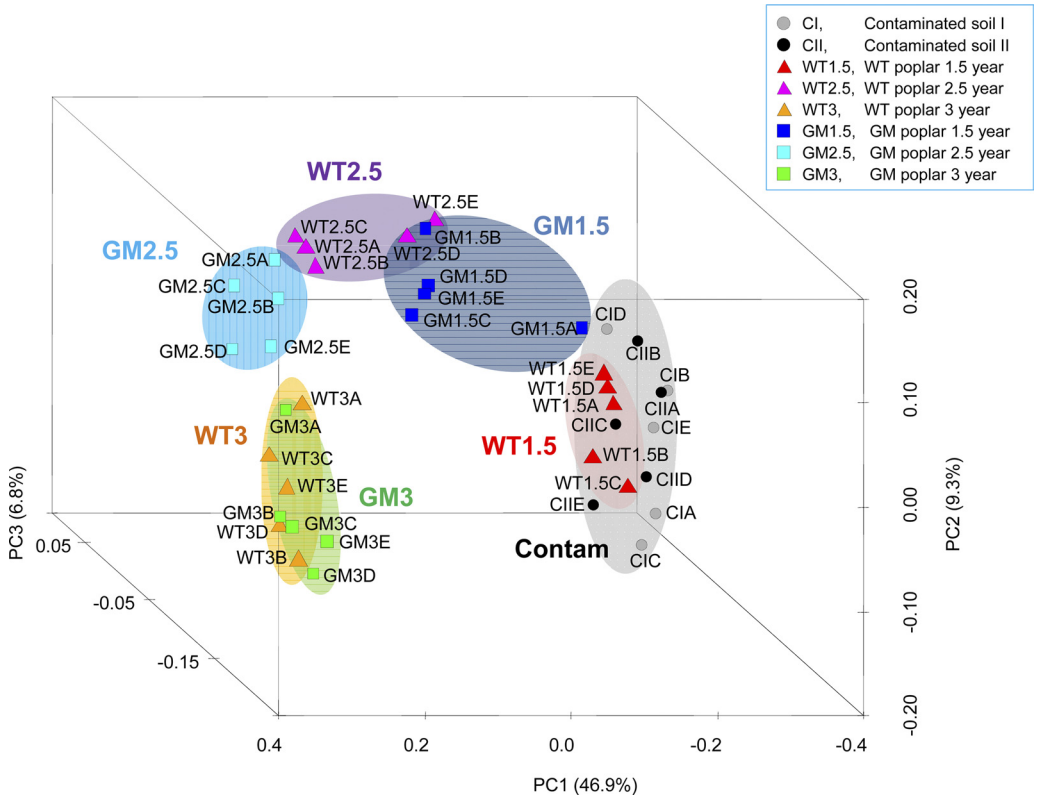

FIG. 2. Principal coordinate analysis (PCoA) of the bacterial community from the 40 individual soil samples. The weighted pairwise UniFrac distances were used for the distance matrix.

pattern of archaeal DNA sequences composition change was similar to bacterial change (Fig. 1b and see Fig. SA1b in the supplemental material). The indicator taxon of contaminated soil was *Euryarchaeota* (46.8%, $P = 0.061$), which was mainly composed of two genus-level clusters, *Thermoplasmatales* DQ303253 (30.8%, $P = 0.127$) and *Thermoplasmatales* EF446196 (11.5%, $P = 0.120$). These two clusters decreased with the advance of poplar growth stages and completely disappeared in the middle growth stage. However, a small portion (0.3 to 2.2%) was recovered at the late stage. The phylum *Thaumarchaeota* was the dominant (77.8 to 99.9%, $P = 0.116$) taxon in poplar-planted soils. The two genus-level clusters *Thaumarchaeota* EU309866 and *Thaumarchaeota* EU155997 increased with the advance of poplar stages ($P = 0.120$ to 0.127), and completely dominated (91.7 to 95.7%) in the late stage. The cluster *Thaumarchaeota* AB050227 was the characteristic genus of the middle stage, representing 72.8% of the population in GM poplar soils aged 1.5 years.

Soil physicochemical properties. The soil physiochemical properties are summarized in Table 2. The overall composition of the soil samples was silt loam. Among the analyzed soil properties, only pH value showed a linear relationship with poplar age ($R^2 = 0.649$). Significant differences ($P < 0.001$) among three soil types, i.e., contaminated, WT poplar-planted and GM poplar-planted soils, were observed in pH, TC, TN, Cd, and Zn but not in EC, C/N, Pb, or As (data not shown).

Strong linear relationships were observed between the concentration of Zn and Cd ($R^2 = 0.996$) and also between the concentration of As and Pb ($R^2 = 0.871$). The correlations of metal concentration for the other metal compounds were

TABLE 2. Summary of soil physicochemical properties[a]

Site	Description	EC	pH	TC (%)	TN (%)	C/N	Cd (mg/kg)	Pb (mg/kg)	As (mg/kg)	Zn (mg/kg)
CI	Contaminated soil set I	595 (524)	3.2 (0.5)	0.14 (0.03)	0.018 (0.004)	8.2 (3.5)	341 (20)	1,257 (39)	1.45 (1.40)	169 (15)
CII	Contaminated soil set II	1,126 (1,015)	2.9 (0.3)	0.35 (0.11)	0.023 (0.004)	15.4 (5.4)	497 (34)	2,169 (69)	2.11 (1.66)	261 (20)
WT1.5	WT poplar, 1.5 yr	1,169 (689)	2.8 (0.3)	0.49 (0.04)	0.038 (0.003)	13.3 (0.9)	498 (17)	2,040 (36)	2.19 (1.39)	270 (15)
WT2.5	WT poplar, 2.5 yr	2,180 (171)	4.6 (0.7)	1.11 (0.08)	0.081 (0.011)	14.3 (2.8)	487 (23)	2,401 (51)	8.87 (2.46)	1,175 (29)
WT3	WT poplar, 3 yr	342 (534)	6.0 (1.1)	0.69 (0.27)	0.064 (0.025)	11.1 (0.6)	158 (9)	721 (17)	3.30 (1.39)	444 (16)
GM1.5	GM poplar, 1.5 yr	1,457 (796)	4.0 (0.5)	1.18 (0.51)	0.060 (0.005)	25.4 (18.0)	791 (27)	3,587 (59)	13.52 (3.41)	2,053 (42)
GM2.5	GM poplar, 2.5 yr	1,665 (902)	5.2 (0.4)	1.13 (0.16)	0.064 (0.005)	18.2 (3.3)	579 (20)	3,455 (47)	12.55 (3.00)	1,892 (35)
GM3	GM poplar, 3 yr	496 (963)	6.9 (0.6)	0.65 (0.45)	0.039 (0.008)	18.0 (14.6)	340 (9)	2,288 (18)	8.23 (1.49)	1,246 (17)

[a] The values are the arithmetic average for each of the eight sites. Each site contains five individual soil samples. Numbers in parentheses are standard deviations.

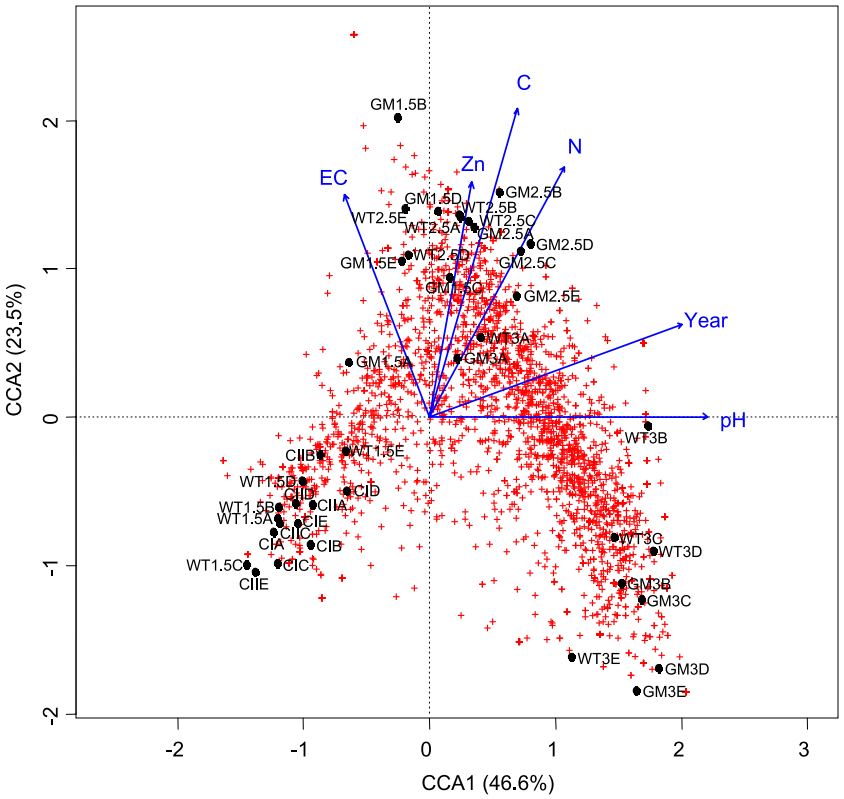

FIG. 3. Canonical correspondence analysis (CCA) of bacterial species abundance and soil features. The percentages of variation explained by each axis are shown. The solid circles indicate samples, and the red crosses indicate bacterial species.

lower than those for the Zn-Cd and As-Pb pairs, but an overall positive linear regression was observed (R^2 = 0.607 to 0.761). The lowest metal concentration was found in the nonphytoremediated control soil. The highest rhizosphere metal concentration was observed in the 2.5-year WT and 1.5-year GM tree soils. The value increased with poplar growth, and then decreased back to the control levels in the late stages of poplar growth (see Fig. SA2a and b in the supplemental material). The maximum metal concentration of the GM rhizosphere was ~1.6-fold higher than that of the WT.

The overall changing pattern of total carbon (TC), total nitrogen (TN), and electrical conductivity (EC) was dependent on poplar growth stage, which was similar to that recorded for changes in metal levels (see Fig. SA2c and d in the supplemental material). The highest values were observed at 2.5 years for WT and 1.5 years for GM but, unlike metals, the difference in maximum values between WT and GM was not evident for TC, TN, or EC.

Correlations between bacterial community structure and environmental factors. To delineate the effect of soil properties on the microbial communities, the geochemical variables were analyzed using CCA for correlation with bacterial community structure. The variables As, Cd, and Pb were removed from the analysis because of their multicollinearity with Zn. The seven soil sites were well separated by CCA, showing a significant correlation between bacterial community structure and environmental factors (Fig. 3). The first canonical axis explained 46.6% of the detected microbial diversity and was positively correlated with pH and poplar age. The second axis represented 23.5% of variance and was positively correlated with Zn, EC, C, and N. Overall, the CCA biplot indicated that the seven soil sites were differentiated mainly with respect to pH and poplar age, but the intermediate time points were additionally distinguished from early or late stage groups by high metal concentrations, total carbon and nitrogen, and electrical conductivity. Thus, while pH was the fundamental regulator, the structure of the microbial community of the rhizosphere was also influenced by metal concentration, total carbon and nitrogen content, and EC.

The PCoA plot of the pairwise UniFrac distance ordination supported the strong influence of pH on the rhizosphere bacterial community structure. The variability in soil bacterial community composition was strongly related to pH (see Fig. SA3a in the supplemental material). The influence of soil pH was also evident in an aspect of bacterial diversity, because a significant correlation between the number of species and pH values was observed (R^2 = 0.915; see Fig. SA3b in the supplemental material), but no significant relationship with any of the other soil characteristics was identified.

DISCUSSION

The structural change of the microbial community was directional, and the speed of change was faster in GM poplars than in WT poplars, as shown in Fig. 2. For both GM and WT poplars, the microbial community of poplars started separating

from that of the control soil in the early stage of poplar cultivation (1.5 years), advanced to the middle-stage group (2.5 years), and finally reached the late-stage group (3 years), the composition of which was very different from that of the contaminated soil community. Interestingly, the rate of microbial community change was slower in WT poplars than in GM poplars. At 1.5 years, the WT community continued to overlap with the contaminated soil site, while the GM community was clearly separated from that of contaminated soils. The composition of the WT 2.5-year microbial community was more advanced than that of the GM 1.5-year community but was still not comparable to that of the GM 2.5-year community. However, the WT 3-year community was eventually similar to the GM 3-year community, implying the stabilization of microbial community features in 3-year-old poplar soils. This phenomenon possibly occurs because of the more active metal uptake ability of GM poplars compared to WT poplars, which resulted in faster changes in the soil environment and hence the microbial habitat.

Several previous studies describing the effects of GMOs on the structure of microbial communities have shown that the effect is temporary and depends on the presence or absence of transgenic plants. For example, seasonal variation in the microbial community structure associated with GM plants has been shown, with no persistence into the next field season, at least according to studies on GM canola and potato (17, 27). Variable differences in the microbial communities between GM and WT poplars were observed in the present study, which were dependent on the stages of poplar growth, and did not continue into the late growth stage. Thus, the results of the present study may also correspond to the transient nature of the GMO effect, as recorded in previous studies. This is probably because the exudation patterns of roots vary according to plant developmental stage and plant type, while the exudation pattern is not different at the fully developed stage. However, further studies using poplars aged 4 years and above are required to make robust conclusions about the effects of GM poplar on the microbial community.

The proliferation of bacterial candidate division AD3 (8.6%) in metal-contaminated soils was remarkable, because such high proportions of AD3 have not been previously reported. This phylum is a candidate division that has only been detected in very small proportions (maximum, 2%) in metagenomic clone libraries (53, 73). The previously reported uncultured clones belonging to this phylum were isolated from tundra soils (10, 53, 54), soil iron-manganese nodules (32), geothermal soils (64), 45,000-year-old soils (65), rainfall site soils (11), watershed soils (31), and plant rhizospheres (45). The low pH and/or high concentration of metals present in mine tailings seem to be responsible for the unprecedented prevalence of AD3, but the reason for this remains unclear at this stage. Since only 16S rRNA gene sequences are known for this division, further studies of enrichment, isolation, and/or single cell genomics using the characteristic soil samples obtained in the present study would broaden our knowledge about this unknown bacterial phylum.

The bacterial and archaeal lineages that have been mostly reported from metal-contaminated acidic environments include *Leptospirillum*, *Acidithiobacillus*, *Thermoplasma*, and *Ferroplasma* (33, 49, 69). The prevalence of species of the genera *Leptospirillum* and *Acidithiobacillus* in metal-contaminated soil was also observed here. This may be because species of these two genera are iron reducers that use metal ions as an energy resource and thus occupy a major portion of the microbial community in low pH environments (36). The other newly detected bacterial indicators of metal contamination identified here are *Planctomyceteceae* DQ906072 and *Xanthomonadales* FJ228294. The clone sequences of accession numbers DQ906072 and FJ228294 originate from the rhizosphere of plants adapted to acid mine drainage (50) and acidic mine pit lakes (www.ncbi.nlm.hih.gov), respectively. This supports our results, implying the presence of two unknown bacterial species that predominantly inhabit metal-contaminated acidic soils. In contrast, the previously reported archaeal genera, namely, *Thermoplasma* and *Ferroplasma*, were not detected. Instead, two genus-level clusters named *Thermoplasmatales* DQ303253 (30.8%) and *Thermoplasmatales* EF446196 (11.5%) were observed as indicator archaea of metal-contaminated soils. Actually, the clone sequences of accession numbers DQ303253 and EF446196 have been reported in extremely acidic rivers with high metal concentrations (23). However, cultured strains are not available for these two sequence clusters, with the results obtained in the present study strongly indicating the presence of novel acidophilic metal-reducing archaeal lineages.

The indicator bacterial and archaeal lineages observed here disappeared during the process of phytoremediation. Instead, well-known heterotrophic bacterial lineages, such as *Arthrobacter* and *Nocardioides*, proliferated, and overall bacterial diversity increased. The two genus-level archaeal clusters *Thaumarchaeota* EU309866 and *Thaumarchaeota* EU155997 increased with the advance of poplar growth stages and were completely dominant (91.7 to 95.7%) in the 3-year-old poplars. The clone sequence of accession numbers EU309866 and EU155997 originated from rhizospheres of the freshwater macrophyte *Littorellauniflora* (34) and minetrophic fen (4), respectively. Thus, the two unknown archaeal clusters observed in the present study were assumed to be heterotrophs that specifically inhabit the rhizosphere.

The evident effect of phytoremediation on acid mine tailings was the neutralization of soil pH. The increment in pH possibly occurred due to the uptake of oxyanion-form metals by poplars, in addition to the release of OH^- into the rhizosphere by the plants, to balance charges after the uptake of metals (24). Furthermore, the increased amount of organic compounds produced by vegetation may be attributed to the improvement of soil condition for neutrophilic and heterotrophic microorganisms, instead of acidophilic autotrophs (25, 49). The linear increment of pH with the aging of poplars was clearly accompanied by an increase in rhizosphere bacterial diversity and changes in community structure. These results are congruent with previous reports suggesting the fundamental role of pH in controlling soil microbial community and diversity (19, 41).

The observed fluctuation of rhizosphere metal concentration in the present study was interesting. The metal concentration was lowest in the nonphytoremediated control soil. In poplar-planted soils, metal concentrations first increased with poplar age and were highest in intermediate time points and then decreased back to control levels in older poplars. A possible explanation for this variation maybe found in previous

studies on the phytoremediation of As using the *Pteris* fern (20, 24). It was reported that the concentration of soluble As is 20 to 40% higher in the rhizosphere compared to bulk soil, because metal-solubilizing exudates produced from plant roots increase As solubility in the rhizosphere (24). In comparison, the total As concentration did not significantly decrease in rhizosphere soil after 1 cropping, despite the substantial removal of As by the plants (20). This is because of the large buffering capacity of soil. Thus, the elevated metal concentrations in the actively growing poplar rhizosphere observed in the present study may be a result of the high concentration of solubilized metals produced from root exudates, while insoluble metal concentrations may be preserved by the resupply of depleted metals from bulk soil. However, further physicochemical study is required to discover the clear reasons of this. Interestingly, the maximum metal concentration of the GM rhizosphere was ~1.6-fold higher than that of the WT rhizosphere. This phenomenon may imply that GM poplar possibly has more active metal solubilization and uptake ability than WT poplar.

The fluctuation of TC, TN, and EC was similar to that observed for metal concentrations. This type of high correlation between environmental factors in the rhizosphere has also been previously reported for dissolved organic carbon (DOC) and water-soluble metals in the rhizosphere (24). The increment of DOC in the rhizosphere (20, 24), as well as elevated TC and TN in bulk soils (22), have been documented in vegetated soils and are congruent with our data. This may be caused by greater metal concentrations in the soil resulting in greater DOC concentrations by the metal-induced plant release of root exudates, plant litter, and soil humus (24). The high content of organic matter produced by the actively metabolizing microbial community interacting with the actively growing poplar rhizosphere could therefore be another major source of increased C, N, and EC.

In conclusion, the shift in the microbial community structure to the late stage was driven faster by the effect of GM phytoremediation than WT phytoremediation. This was possibly because of the more active metal uptake ability of GM plants, which accelerated the pH-neutralizing effect on the rhizosphere soil environment compared to WT plants. The present study contributes toward improving our understanding of the effect of GM phytoremediation in the aspect of microbe-plant interactions.

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